

Suppressive Effect of IL-4 on IL-13-Induced Genes in Mouse Lung¹

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Although IL-4 signals through two receptors, IL-4R α /common γ -chain (γ_c) and IL-4R α /IL-13R α 1, and only the latter is also activated by IL-13, IL-13 contributes more than IL-4 to goblet cell hyperplasia and airway hyperresponsiveness in murine asthma. To determine whether unique gene induction by IL-13 might contribute to its greater proasthmatic effects, mice were inoculated intratracheally with IL-4 or IL-13, and pulmonary gene induction was compared by gene microarray and real-time PCR. Only the collagen α_2 type VI (Ca2T6) gene and three small proline-rich protein (SPRR) genes were reproducibly induced >4-fold more by IL-13 than by IL-4. Preferential IL-13 gene induction was not attributable to B cells, T cells, or differences in cytokine potency. IL-4 signaling through IL-4R α / γ_c suppresses Ca2T6 and SPRR gene expression in normal mice and induces these genes in RAG2/ γ_c -deficient mice. Although IL-4, but not IL-13, induces IL-12 and IFN- γ , which suppress many effects of IL-4, IL-12 suppresses only the Ca2T6 gene, and IL-4-induced IFN- γ production does not suppress the Ca2T6 or SPRR genes. Thus, IL-4 induces genes in addition to IL-12 that suppress STAT6-mediated SPRR gene induction. These results provide a potential explanation for the dominant role of IL-13 in induction of goblet cell hyperplasia and airway hyperresponsiveness in asthma. *The Journal of Immunology*, 2005, 174: 4630–4638.

Thelper 2 cytokines are overexpressed in the lungs of asthmatic individuals and are required for the induction of allergic airway disease in mouse models of asthma (1–16). Although each Th2 cytokine contributes to asthma pathogenesis, two related cytokines, IL-4 and IL-13, have a dominant role (7, 17–24). This role has been demonstrated most clearly in mouse models, in which disease is suppressed by reagents that block both cytokines; by reagents that inhibit IL-4 and IL-13 binding to IL-4R α , the signaling polypeptide in the IL-4Rs/IL-13Rs; and by genetic deletion of STAT6, a transcription factor that is activated selectively by IL-4 and IL-13 (21, 25, 26). Consistent with IL-4/IL-13 activation of a common signaling pathway, inhalation or pulmonary expression of either cytokine induces features characteristic of asthma, including airway hyperresponsiveness (AHR),³ eosinophil infiltration, collagen deposition, and goblet cell hyperplasia (17, 18, 23, 27, 28).

The pulmonary effects of IL-4 and IL-13 are not, however, identical. IL-4 has a greater effect than IL-13 on the induction of IgE responses, Th2 cytokine production, and pulmonary eosinophilia,

in mice immunized nasally or intratracheally (i.t.) with allergens (18, 29, 30). This most likely reflects the selective activation by IL-4, but not IL-13 of the type 1 IL-4R, which is composed of IL-4R α and the cytokine receptor common γ -chain (γ_c) and is expressed on several bone marrow-derived cell types (31–33). In contrast, both IL-4 and IL-13 activate the type 2 IL-4R, which is composed of IL-4R α and IL-13R α 1 and is expressed on multiple cell types, but not on murine B or T lymphocytes (34). Although these considerations might suggest that the effects of IL-13 would be redundant in asthma, studies of allergen-induced murine asthma have shown that IL-13 is considerably more important than IL-4 in the induction of AHR, pulmonary fibrosis, and goblet cell hyperplasia (17, 18, 35). To some extent, this may reflect greater pulmonary expression of IL-13 than IL-4 in mouse models of asthma. However, the development of severe pulmonary fibrosis and AHR by mice in which a Clara cell promoter (CC-10) induces overexpression of an IL-13 transgene selectively in the lungs, but not by mice in which the same promoter induces IL-4 overexpression (28, 36), suggested to us that IL-13 might have effects on pulmonary gene expression that are not shared by IL-4. To investigate this possibility, we performed a gene scan that compared pulmonary gene expression in mice that had repeatedly inhaled saline, IL-4, or IL-13, and used real-time RT-PCR to confirm pertinent results. These studies demonstrate that IL-13 indeed has unique effects on pulmonary gene expression and suggest that these unique effects result from IL-4 signaling through the type 1 IL-4R that limits the Th2 response.

Materials and Methods

Mice

Mice were obtained or purchased from the following sources and were bred in vivariums at the Cincinnati Veterans Administration Medical Center and Cincinnati Children's Hospital Medical Center: BALB/c (National Cancer Institute); C57BL/10 (Taconic Farms); RAG2-deficient mice on a C57BL/10 background (Taconic Farms); RAG2/ γ_c -double-deficient mice on a C57BL/10 background (Taconic Farms); and IL-13R α 2-deficient

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; γ_c , common γ -chain; Ca2T6, collagen α_2 type VI; Ct, crossing point; DC, dendritic cell; IL-4C, IL-4/anti-IL-4 mAb complex; i.t., intratracheal; SPRR, small proline-rich protein.

mice (37) and background-matched control mice (D. Donaldson, Wyeth-Genetics Institute, Cambridge, MA). Mice that express an IL-4 transgene under the regulation of a lung-specific CC-10 promoter (27) were obtained from J. Whitsett (Cincinnati Children's Hospital Medical Center, Cincinnati, OH) and bred onto a BALB/c background. Control mice were on the identical background. Mice that express a tetracycline-inducible IL-13 transgene under the regulation of the CC-10 promoter have been described previously (38). STAT4-deficient (39) and STAT6-deficient mice (40) on a BALB/c background were obtained from M. Grusby (Harvard University, Cambridge, MA).

Reagents

IL-4 was purchased from PeproTech; IL-13 was a gift of D. Donaldson; rat IgG2b anti-mouse IL-4 mAb (BVD4-1D11) (41) was obtained from the American Type Culture Collection with the permission of DNAX and the help of R. Coffman (DNAX, Palo Alto, CA). IL-4 contained <1 EU/ml; IL-13 contained <2 EU of LPS/ml. Hybridomas that secrete a neutralizing rat IgG1 anti-mouse IFN- γ mAb (XMG-6) (42) or an isotype-matched control mAb (GL113) were originally obtained from R. Coffman and J. Abrams (DNAX, Palo Alto, CA).

IL-4/Anti-IL-4 complexes

To extend the in vivo $t_{1/2}$ of IL-4, mice were inoculated in some experiments with complexes produced by mixing mouse rIL-4 with a neutralizing anti-IL-4 mAb (BVD4-1D11) at a 2:1 molar (1:5 weight) ratio, which saturates the mAb with IL-4. We have previously demonstrated that these complexes have an in vivo $t_{1/2}$ of ~1 day and slowly dissociate, releasing biologically active IL-4 (43). Because these complexes contain a single IgG molecule, they neither fix complement nor react more avidly than uncomplexed serum IgG with Fc γ Rs. Because the IL-4 in these complexes is unable to bind to IL-4Rs, there is no possibility for these complexes to mediate cross-linking of IL-4Rs and Fc γ Rs.

Intratracheal inoculation

Mice were anesthetized by i.p. injection of ketamine and xylazine and allowed to hang vertically with their mouths open, supported by a taut string placed under their canine teeth. Their tongues were gently withdrawn with a blunt forceps to keep them from swallowing, and 40 μ l of saline $\pm$ cytokine was pipetted onto the base of the tongue. When the mice had aspirated the pipetted solution, they were placed on their sides in a box flushed with 100% oxygen until they recovered from the anesthesia.

Gene scans

Whole lung gene expression patterns in mice that had inhaled PBS, IL-4, or IL-13 (four/group) were compared using Affymetrix microarrays. Lungs were frozen in liquid nitrogen immediately after harvest and homogenized in TRIzol reagent (Invitrogen Life Technologies) using an ULTRA-TUR-RAX power homogenizer. Total RNA was isolated with the TRIzol reagent method, followed by RNeasy purification (Qiagen). A total of 50 μ g of cleaned total RNA was processed for double-stranded cDNA production using a T7 promoter-tailed oligo(dT) primer from SuperScript Choice system (Invitrogen Life Technologies). Biotinylated cRNA was produced using the ENZO Bioarray RNA transcript labeling kit (Qiagen), and fragmented randomly to ~200 bp (200 mM Tris-acetate, pH 8.2, 500 mM KOAc, 150 mM MgOAc). Equal amounts of cRNA from two of the four mice in each experimental group (PBS, IL-4/anti-IL-4 mAb complex (IL-4C), IL-13, IL-4C + IL-13) were pooled, producing two distinct pools for each experimental group. cRNA for each pool was hybridized on Affymetrix MG-U74 v2 Microarray chips, which contain oligonucleotide probe sets representing 12,422 genetic elements, for 16 h in an Affymetrix Hybridization Oven 640 (Affymetrix). Microarray chips were washed and stained with PE-streptavidin on the Affymetrix Fluidics Station 400 using instructions and reagents provided by Affymetrix. The stained arrays were scanned using the Hewlett-Packard G2500A Gene Array Scanner (Hewlett-Packard) at a wavelength of 488 nm. Gene transcript levels were determined from the data image files using algorithms in the Microarray Suite Version 5 software (Affymetrix). Differences between treatment groups were determined using the GeneSpring software (Silicon Genetics). Data for each cytokine treatment group were normalized to the average of the PBS-treated groups. Subsequently, differential gene expression between cytokine treatment groups was assessed by filtering the expression data for those genes that met the criteria of >4-fold increased or decreased relative to the cytokine treatment of interest.

Quantitative real-time RT-PCR

Lungs were frozen in liquid nitrogen immediately after harvest and homogenized in 2 ml of TRIzol reagent (Invitrogen Life Technologies). Total RNA was isolated, as recommended by the manufacturer. Before cDNA synthesis, each RNA sample was treated with DNase I (Invitrogen Life Technologies) to eliminate any potential contamination with genomic DNA. Reverse transcription was performed on 2 μ g of total RNA/sample using Superscript II (RNase H⁻; Invitrogen Life Technologies) and random hexamers (Roche) as the first-strand cDNA primer. Real-time PCR analysis was conducted in duplicate in the iCycler (Bio-Rad) real-time PCR machine, using Bio-Rad's iQ SYBR Green Supermix Taq polymerase mix. Primer concentrations, annealing temperatures, and cycle number were optimized for each primer pair. The reaction mixture contained (20 μ l final volume): 2 or 4 μ l of cDNA (corresponding to 33 ng of total RNA from the reverse-transcription reaction), 1–2 μ M sense primer, 1–2 μ M antisense primer, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM each dNTP (dATP, dCTP, dGTP, and dTTP), 25 U/ml iTaq DNA polymerase, 3 mM MgCl₂, SYBR Green I, and 10 nM fluorescein. Melting curve analyses and negative controls were included in each assay to ensure that PCR product was not the result of reaction contamination. Real-time PCR primer pairs were designed using Beacon Designer software and were as follows: collagen α_2 type VI (Ca2T6) (NM_146007) sense GCACTCTAT GCGTAAGC, antisense CCCAAGGTGACCGTCT; small proline-rich protein 1a (SPRR1a) (NM_009264) sense GAGTATTAGGACCAAGT GCT, antisense GGGCACAAGGTTCTCTG; SPRR2a (NM_011468) sense TGGGCTTGTCGTCCTGTCATG, antisense ATGGCTGAGGTGGGC ATTGCTC; SPRR2b (Celera mC120583) sense GCCCATACAGGGA GAT, antisense GCTTGAGTACCAGGAATACT; IFN- γ (NM_008337) sense TCAAGTGGCATAGATGTGGAAGAA, antisense TGGCTCTG CAGGATTTTCATG; and β -actin (NM_007393) sense GTGACGTTGA CATCCG, antisense CAGTAACAGTCCGCCT. For each primer pair, a dilution curve of a positive cDNA sample was included to enable calculation of the efficiency of the amplification. The relative message levels of each target gene were normalized to the housekeeping gene, β -actin, using the method described previously (44). Briefly, the crossing points for each target gene were normalized to the crossing point of the housekeeping gene by the formula: relative expression = $((E_{ref})^{Ct_{ref}}) \times 100,000 / ((E_{target})^{Ct_{target}})$, where E_{ref} , E_{target} , Ct_{ref} , and Ct_{target} equal the efficiencies and crossing points of the housekeeping gene and the target gene, respectively. Although the ratios of calculated gene expression between treatment groups were quite reproducible from one experiment to another, the absolute values of calculated gene expression sometimes varied in different experiments, even when the same gene was evaluated following the same treatment of mice of the same strain. This variation appeared primarily to be related to the method by which the real-time PCR software calculated the Ct for each sample relative to the range of fluorescence baseline values in each experiment. For this reason, message level numbers are provided only as a general indicator of relative gene expression.

Statistics

Data were analyzed by ANOVA and Fisher's protected least significant difference for statistical significance, using Statview. Values of $p < 0.05$ were considered statistically significant.

Results

Gene scans of lungs from mice inoculated i.t. with PBS, IL-4C, or IL-13

To evaluate whether there are differences in the abilities of inhaled IL-4 and IL-13 to induce the expression of specific genes in lung cells, BALB/c female mice were inoculated daily i.t. with PBS for 10 days, IL-4C that contained 2 μ g of IL-4 for 10 days, or PBS for 7 days, followed by IL-13 for 3 days. This asymmetric screening protocol was based on doses of IL-4C or IL-13 that induced similar increases in AHR, pulmonary eosinophilia, and pulmonary goblet cell hyperplasia (data not shown). IL-4C was used instead of free IL-4 because previous studies had demonstrated that IL-4C is considerably more potent than free IL-4 at inducing pulmonary eosinophilia (data not shown). Gene scans of lungs from these mice revealed 116 genes with expression increased at least 6.5-fold with IL-4 or IL-13 vs PBS. Of these, expression of 64 genes was increased at least 7-fold with IL-4 vs PBS; expression of 31 genes was increased at least 7-fold with IL-13 vs PBS; expression of 21

Table I. Genes expressed in the lungs following inhalation of IL-13, but not IL-4

Gene	Function
M26071	Coagulation factor III/cds
U77462	Eotaxin precursor (Scya11) ^a
AI314692	ATP-sensitive inward rectifier potassium channel 14 ^a
AA591002	Sodium/glucose cotransporter ^a
AB016592	Gob-4 ^a
AJ224761	Lysine-ketoglutarate reductase ^a
X61597	Kallikrein-binding protein ^a
Procollagen, type VI, α_2 ^a	
SPRR1a	
SPRR2A/cds SPRR2a gene ^a	
SPRR2B/cds SPRR2b gene ^a	

^a Also up-regulated in IL-13 vs PBS, not IL-4 vs PBS.

genes was increased at least 7.5-fold with IL-4 vs IL-13; and expression of 11 genes (Table I) was increased at least 7-fold with IL-13 vs IL-4. The high (6.5- to 7.5-fold) cutoff used in this analysis was chosen to limit the number of genes subjected to further evaluation and to focus on those genes that showed the most robust differences. Because increased gene expression in response to IL-4 vs IL-13 is easily explainable by the ability of IL-4, but not IL-13, to signal through the type 1 IL-4R (IL-4R α / γ_c) (33), while increased gene expression in response to IL-13 vs IL-4 is less easily explained, we focused our studies on genes induced more by IL-13 than by IL-4.

Confirmation of gene scan data by real-time RT-PCR

RNA from the same experiment was studied by real-time RT-PCR. Of the 11 genes that were induced considerably more by IL-13 than IL-4 in the gene scan, real-time RT-PCR determined that all except Gob-4, SCYA11, Kallikrein binding protein, and coagulation factor III were induced at least 4-fold more by IL-13 than IL-4. In two additional experiments, one which inoculated C57BL/10 mice i.t. with saline, IL-4C that contained 2 μ g of IL-4, or 5 μ g of IL-13 for 3 consecutive days, the other that inoculated BALB/c mice i.t. with saline, IL-4C or IL-13 for 4 consecutive days, 4 of these 7 genes (SPRR1a, SPRR2a, SPRR2b, and Ca2T6) were again induced at least 4-fold more by IL-13 than by IL-4, whereas the relative IL-4/IL-13-induced expression of the other 3 genes was variable (data not shown). Consequently, subsequent studies were restricted to Ca2T6 and the three SPRR genes.

Dose dependence of IL-13 vs IL-4 gene induction

Because our initial gene scan treated mice with different doses of IL-4 vs IL-13 and used Ab-complexed IL-4, but uncomplexed IL-13, we performed dose-response studies that also compared the effects on gene expression of free IL-4 vs IL-4C to evaluate whether either factor might account for selective induction of the Ca2T6 and SPRR genes (Fig. 1). Repeated inhalation of 5 μ g of IL-13 induced much stronger Ca2T6 and SPRR gene expression than did repeated inhalation of up to 8 μ g of free IL-4 or IL-4C that contained up to 8 μ g of IL-4 (Fig. 1A), and repeated inhalation of as little as 0.3 μ g of IL-13 induced stronger Ca2T6 and SPRR gene expression than repeated inhalation of IL-4C that contained 2 μ g of IL-4 (Fig. 1B). Free IL-4 and IL-4C that contained an equal amount of IL-4 had nearly identical effects on gene expression (Fig. 1A). Thus, the selective ability of IL-13 to strongly induce pulmonary Ca2T6 and SPRR gene expression is not explained by putative differences in the biological effects of free vs complexed IL-4 or by concentration-dependent effects of IL-4 or IL-13.

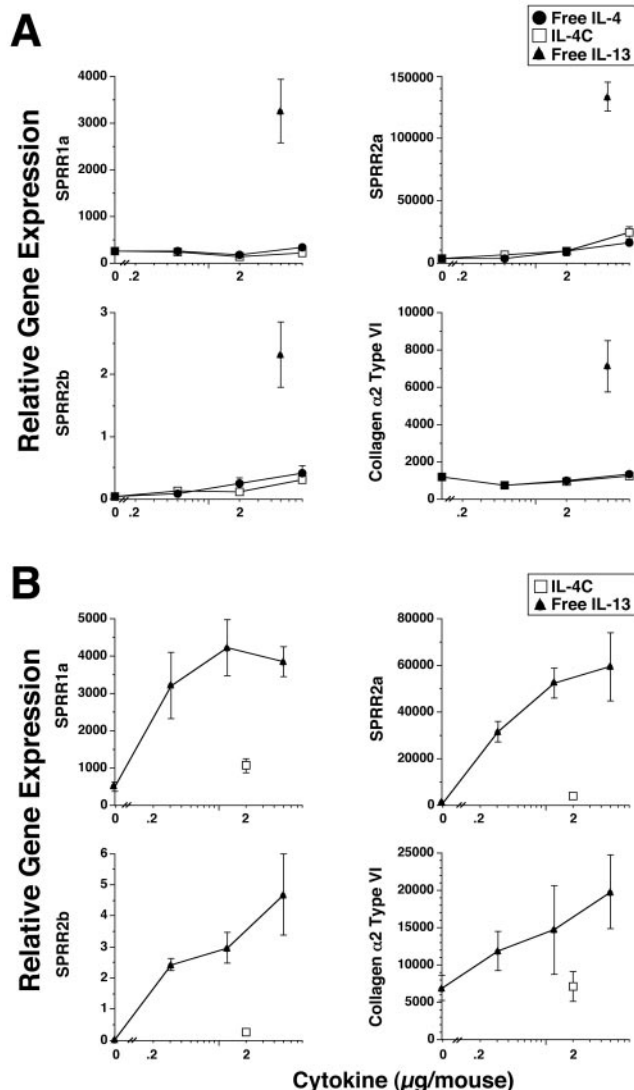


FIGURE 1. Dose dependence of IL-13-selective gene induction. Anesthetized BALB/c mice (five/group) were inoculated daily i.t. with saline, free IL-4, IL-4C, or IL-13, at the doses shown, for 7 days. Mice were sacrificed 1 day after the last i.t. inoculation. RNA prepared from their lungs was reverse transcribed and analyzed for SPRR1a, SPRR2a, SPRR2b, and Ca2T6 cDNA levels by real-time PCR. Separate experiments are shown in A and B.

Induction of Ca2T6 and SPRR gene expression by IL-13 is IL-4R α specific and STAT6 dependent

The ability of IL-13, but not IL-4, to induce specific genes might be explained by IL-13 induction of these genes through a mechanism other than the IL-4R α /STAT6 signaling pathway that is shared with IL-4 or by induction of these genes by a contaminant in our IL-13 preparation. In either case, IL-13 induction of Ca2T6 and SPRR gene expression would be IL-4R α and STAT6 independent. To investigate these possibilities, we evaluated the abilities of repeatedly inhaled IL-4C and IL-13 to induce pulmonary expression of these genes in IL-4R α -deficient mice (Fig. 2A) and compared the abilities of IL-13 to induce pulmonary expression of these genes in wild-type vs STAT6-deficient mice (Fig. 2B). Results of these studies demonstrated that IL-13 induction of Ca2T6 and SPRR gene expression is completely IL-4R α and STAT6 dependent. Because we could not totally eliminate the possibility that our IL-4 and/or IL-13 preparations might contain contaminants

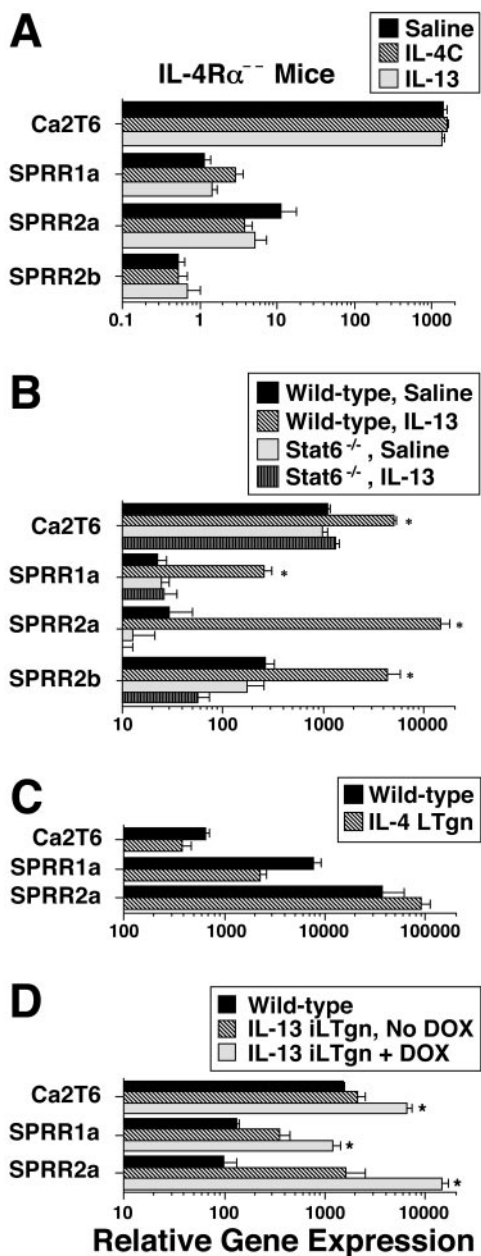


FIGURE 2. IL-13-selective pulmonary gene induction is IL-4R α specific and STAT6 dependent. *A*, Anesthetized BALB/c IL-4R α -deficient mice (five/group) were inoculated daily i.t. with saline, IL-4C that contained 2 μ g of IL-4, or 5 μ g of IL-13 for 4 days. *B*, Anesthetized BALB/c wild-type or STAT6-deficient mice were inoculated daily i.t. with saline or 5 μ g of IL-13 for 3 days. Lungs were analyzed for SPRR1a, SPRR2a, SPRR2b, and Ca2T6 mRNA. *C*, Lungs from wild-type BALB/c mice and BALB/c mice that express a lung-specific CC-10-IL-4 transgene (IL-4 LTgn) were analyzed for SPRR1a, SPRR2a, and Ca2T6 mRNA. *D*, BALB/c mice that express a tetracycline-inducible lung-specific CC10-IL-13 transgene (iIL-13 LTgn) were provided with normal food or food that contained doxycycline (DOX) for 2 wk. Lungs from these mice and wild-type BALB/c mice were analyzed for SPRR1a, SPRR2a, and Ca2T6 mRNA. Note that gene expression in all panels is plotted on a logarithmic scale. *, $p < 0.05$ vs mice treated with saline (*A* and *B*), or vs wild-type mice and iIL-13 LTgn mice provided with food that lacked doxycycline (*D*).

that could act synergistically with IL-4 or IL-13 to inhibit or stimulate induction of the Ca2T6 and SPRR genes, respectively, we also compared Ca2T6 and SPRR gene expression in the lungs of wild-type mice and mice that express a lung-specific IL-4 trans-

gene (Fig. 2C), and in the lungs of wild-type mice and mice that express a tetracycline-inducible lung-specific IL-13 transgene following provision of normal food or food that contained doxycycline (Fig. 2D). We could not directly compare gene expression between the IL-4 lung transgenic mice and the IL-13 lung transgenic mice because the IL-4 transgene is constitutively expressed, while the IL-13 transgene is inducible (although somewhat leaky) and because the two mouse strains were raised in different colonies. Regardless, the results in this experiment confirm our results with mice administered IL-4 or IL-13; the Ca2T6 and SPRR genes were not expressed at a higher level in the IL-4 lung transgenic mice than in the matched wild-type mice, while these genes were expressed at a higher level in the lungs of the IL-13 transgenic mice than in the matched wild-type mice, particularly after the IL-13 transgenic mice were fed doxycycline.

Selective induction of Ca2T6 and SPRR gene expression by IL-13 is IL-13R α 2 independent

IL-4 and IL-13 signaling potentially differ in that only the latter can interact with IL-13R α 2 (34, 37). Even though this molecule is thought to act primarily as a nonsignaling, negative regulator of IL-13, it remained possible that it might participate in a multichain signaling receptor that could selectively respond to IL-13. To test the possibility that IL-13R α 2 is responsible for the selective effects of IL-13, we evaluated the ability of inhaled IL-13 to induce Ca2T6 and SPRR gene expression in wild-type and IL-13R α 2-deficient mice on the same genetic background. To simultaneously evaluate the possibility that IL-13R α 2 might have quantitative, but not qualitative effects on IL-13-induced gene expression, different groups of mice were inoculated with an optimal (1.25 μ g) or a suboptimal (0.31 μ g) dose of IL-13. Because no consistent differences were found between wild-type and IL-13R α 2-deficient mice in IL-13 induction of pulmonary gene expression at either dose (Fig. 3), we conclude that IL-13R α 2 is not required for the unique effects of IL-13.

Kinetics of IL-4- vs IL-13-induced gene expression

Both IL-4 and IL-13 inhalation rapidly induce AHR, while chronic expression of an IL-13 transgene, but not an IL-4 transgene, on a lung-specific CC-10 promoter has been reported to be associated with chronic AHR (27, 28). These observations raised the possibility that IL-4 might initially induce the Ca2T6 and SPRR genes,

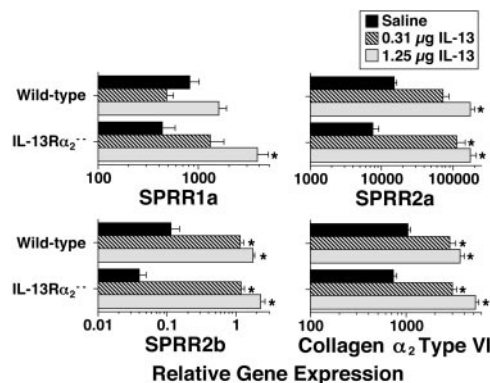


FIGURE 3. IL-13R α 2 does not contribute to expression of IL-13-selective genes. Anesthetized BALB/c wild-type or IL-13R α 2-deficient mice (five/group) were inoculated i.t. with saline, 0.31 μ g/day IL-13 or 1.25 μ g/day IL-13, for 7 days and sacrificed 1 day after the last inoculation. Lungs were analyzed for SPRR1a, SPRR2a, SPRR2b, and Ca2T6 mRNA. Note that gene expression is plotted on a logarithmic scale. *, $p < 0.05$ for same strain treated with saline.

but lack the ability to maintain their expression at elevated levels. Kinetic studies, in which real-time RT-PCR was used to investigate IL-4C vs IL-13 induction of Ca2T6 and the three SPRR genes after 1–7 days of daily i.t. cytokine inoculation, were performed to investigate this possibility (Fig. 4). IL-13 induced increased expression of each of these genes at all time points studied. Although IL-4C inhalation failed to induce Ca2T6 or SPRR1a gene expression at any time point studied, it initially induced SPRR2a and SPRR2b gene expression. SPRR2a and SPRR2b gene expression decreased when mice were treated for a longer period of time with IL-4, even though it increased in mice treated for >1 day with IL-13. These observations are consistent with the possibility that IL-4 might have effects that suppress expression of Ca2T6 and the SPRR genes. In addition, an observation that IL-4, but not IL-13, induced IFN- γ gene expression (Fig. 4) suggested a possible mechanism for selective suppression of SPRR gene expression.

IL-4C inhibits IL-13-induced SPRR gene expression

If IL-4 fails to chronically induce Ca2T6 and SPRR gene expression because it induces other genes that suppress their expression, treatment with IL-4 might be able to inhibit IL-13 induction of the Ca2T6 and SPRR genes. To evaluate this possibility, BALB/c mice were inoculated i.t. with saline or IL-4C that contained 2 μ g of IL-4 daily for 10 days. Some of these mice also were inoculated daily i.t. with 5 μ g of IL-13 for the last 3 days of this 10-day period. Comparison of gene expression in the saline/IL-13-treated mice with gene expression in the IL-4C/IL-13-treated mice (Fig. 5) demonstrated that IL-4C inhalation significantly inhibited IL-13 induction of Ca2T6, SPRR1a, and SPRR2a gene expression, but not SPRR2b gene expression.

IL-4 induction of Ca2T6 and SPRR gene expression is similar in wild-type and RAG2-deficient mice, but selectively increased in mice deficient in both RAG2 and γ_c

IL-4 inhibition of IL-13-induced Ca2T6 and SPRR gene expression raised the possibility that signals induced through the type 1

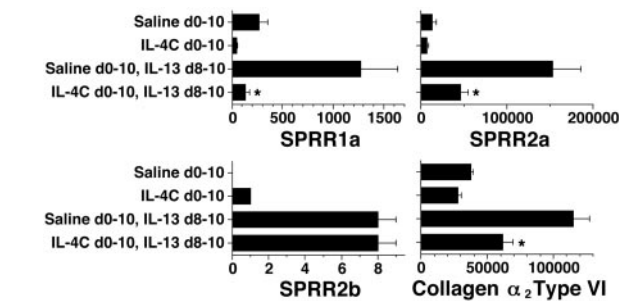


FIGURE 5. IL-4 suppresses IL-13 induction of SPRR genes. Anesthetized BALB/c mice (10/group) were inoculated daily i.t. with saline or IL-4C that contained 2 μ g of IL-4 for 10 days. Groups of these mice were also inoculated daily i.t. with 5 μ g of IL-13 for the last 3 days. Mice were sacrificed 1 day after the last i.t. inoculation. Lungs were analyzed for SPRR1a, SPRR2a, SPRR2b, and Ca2T6 mRNA. *, $p < 0$ vs same strain treated with saline.

IL-4R (IL-4R α/γ_c) might suppress the expression of these genes. Because the type 1 IL-4R is the only IL-4R expressed by murine B and T lymphocytes, it was possible that IL-4 signaling of B or T cells might block the induction of Ca2T6 and SPRR gene expression. To investigate this possibility, we compared the abilities of IL-4C and IL-13 to induce Ca2T6 and SPRR gene expression in C57BL/6 wild-type and RAG2-deficient mice (Fig. 6). No differences were observed between the two mouse strains: the Ca2T6 and SPRR genes were selectively induced by IL-13. This still left open the possibility that IL-4 signaling through the type 1 IL-4R on cells other than B or T cells might suppress Ca2T6 and SPRR gene expression. To investigate this possibility, we compared the Ca2T6 and SPRR gene responses in C57BL/10 wild-type mice and RAG2/ γ_c -double-deficient mice (which are more easily available than γ_c -deficient mice) (Fig. 7). A lower dose of IL-13 (500 ng/day) and a higher dose of IL-4 (10 μ g/day) were used in this experiment than in previous experiments to make any increase in responses to IL-13 in the RAG2/ γ_c -double-deficient mice more apparent and to maximize possible suppressive effects of IL-4. Even at the reduced dose, IL-13 induced similar levels of Ca2T6, SPRR2a, and SPRR2b gene expression in wild-type and RAG2/ γ_c -double-deficient mice. In contrast, IL-4C induced considerably

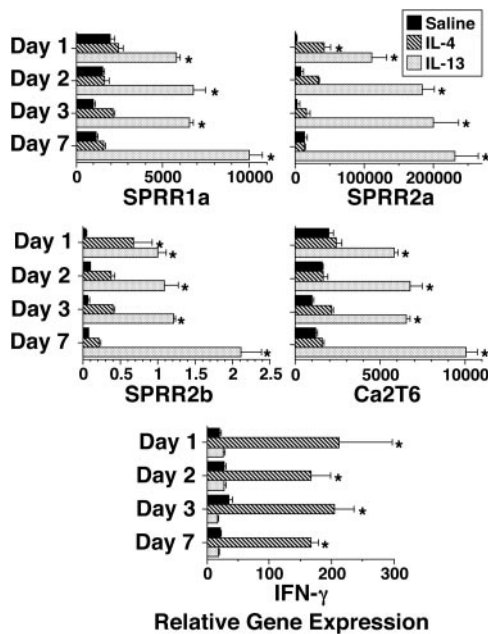


FIGURE 4. Kinetics of IL-13-selective gene induction. Anesthetized BALB/c mice (five/group) were inoculated daily i.t. with saline, IL-4C that contained 2 μ g of IL-4, or 5 μ g of IL-13 for 1, 2, 3, or 7 days. Mice were sacrificed 1 day after the last i.t. inoculation. Lungs were analyzed for SPRR1a, SPRR2a, SPRR2b, and Ca2T6 mRNA.

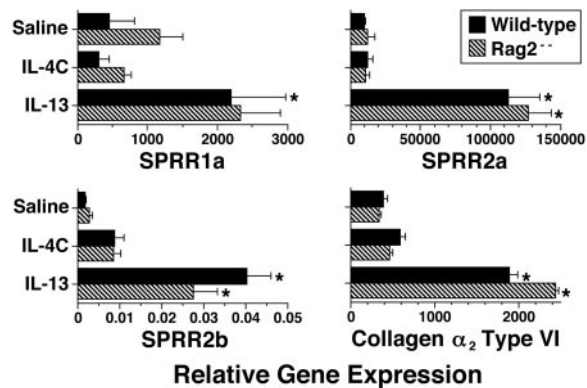


FIGURE 6. Ca2T6 and SPRR genes are selectively induced by IL-13 in RAG2-deficient mice. Anesthetized wild-type or RAG2-deficient mice (five/group) were inoculated daily i.t. for 4 days with saline, IL-4C that contained 2 μ g of IL-4, or 5 μ g of IL-13. Mice were sacrificed 1 day after the last i.t. inoculation. Lungs were analyzed for Ca2T6, SPRR1a, SPRR2a, and SPRR2b mRNA. *, $p < 0.05$ for same strain treated with saline.

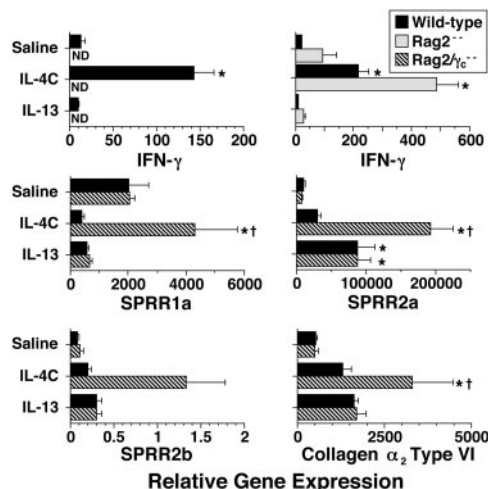


FIGURE 7. IL-4 induction of Ca2T6 and SPRR genes is increased in the absence of γ_c . Anesthetized wild-type, RAG2-deficient, or RAG2/ γ_c -double-deficient mice (five/group) were inoculated daily i.t. for 4 days with saline, IL-4C that contained 2 μ g of IL-4, or 1 μ g of IL-13. Mice were sacrificed 1 day after the last i.t. inoculation. Lungs from wild-type and RAG2/ γ_c -double-deficient mice were analyzed for SPRR1a, SPRR2a, SPRR2b, Ca2T6, and IFN- γ mRNA; lungs from RAG2-deficient mice were analyzed for IFN- γ . *, $p < 0.05$ vs same strain treated with saline. †, $p < 0.05$ for IL-4C-treated wild-type mice vs IL-4C-treated RAG2/ γ_c -deficient mice. ND = not detectable.

greater expression of all four genes in the RAG2/ γ_c -double-deficient mice than in the wild-type mice. Furthermore, IL-4C induced IFN- γ gene expression in wild-type mice and RAG2-deficient mice, but not in RAG2/ γ_c -double-deficient mice, while IL-13 failed to induce IFN- γ expression in any strain tested. Thus, IL-4 signaling through the type 1 IL-4R appears to induce IFN- γ gene expression and suppress type 2 IL-4R-mediated induction of the Ca2T6 and SPRR genes, and neither B cells nor T cells are required for either effect.

IL-4 suppresses Ca2T6 gene expression through STAT4

IL-4, but not IL-13, has been reported to induce dendritic cells (DCs) to secrete IL-12 (45). IL-12, by activating STAT4, stimulates the expression of IFN- γ , a cytokine that suppresses several effects of IL-4 and IL-13 (46–49). To determine whether IL-4 induction of IL-12 might account for selective induction of the Ca2T6 and SPRR genes by IL-13, we compared IL-4 and IL-13 induction of these genes in wild-type vs STAT4-deficient mice (Fig. 8A). IL-13 has similar effects on gene expression in both strains, but IL-4 induction of the Ca2T6 gene was increased in the STAT4-deficient mice. There was also a tendency toward increased SPRR1a and SPRR2a gene expression in these mice, but there was considerable variability of the expression of these genes within these groups and the apparent differences were not statistically significant. Interestingly, IL-4 induced IFN- γ gene expression in both wild-type and STAT4-deficient mice, although it induced somewhat more IFN- γ in the wild-type mice.

In an attempt to confirm the relationship between STAT4 and Ca2T6 gene expression, we evaluated whether inhalation of IL-12 plus IL-18, which stimulate T cell and NK cell IFN- γ expression more potently than IL-12 alone (50–53) would suppress IL-13 induction of the Ca2T6 and/or SPRR genes (Fig. 8B). As expected, inhalation of IL-12 plus IL-18 strongly increased pulmonary IFN- γ gene expression. IL-13 neither induced IFN- γ gene expression nor suppressed IL-12/IL-18 induction of IFN- γ . However, although IL-12 plus IL-18 suppressed IL-13-induced Ca2T6 gene

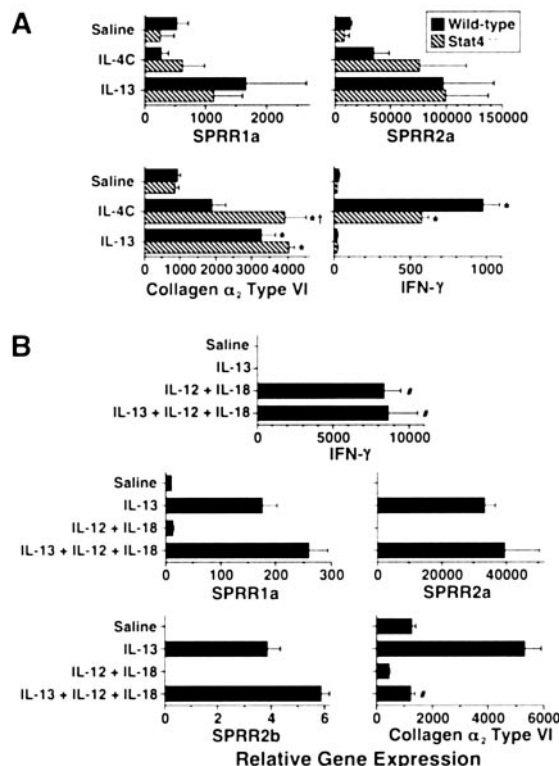


FIGURE 8. IL-4 suppresses Ca2T6 gene expression through STAT4 signaling. **A**, Induction of Ca2T6 gene expression is increased in STAT4-deficient mice. BALB/c wild-type or STAT4-deficient mice (five/group) were inoculated i.t. with saline, IL-4C that contained 10 μ g of IL-4, or 1 μ g of IL-13 daily for 5 consecutive days. Mice were sacrificed 1 day after the last i.t. inoculation. Lungs were analyzed for SPRR1a, SPRR2a, SPRR2b, Ca2T6, and IFN- γ mRNA. SPRR2b levels are not shown because they were too low to be reliably quantitated. *, $p < 0.05$ for same strain treated with saline. †, $p < 0.05$ vs wild-type mice given the same treatment. **B**, IL-13-induced Ca2T6 gene expression is suppressed by a combination of IL-12 and IL-18. BALB/c mice were inoculated i.t. on 4 consecutive days with saline, 5 μ g of IL-13, 100 ng of IL-12 + 500 ng of IL-18, or 5 μ g of IL-13 + 100 ng of IL-12 + 500 ng of IL-18. Mice were sacrificed 1 day after the last i.t. inoculation. Lungs were analyzed for SPRR1a, SPRR2a, SPRR2b, Ca2T6, and IFN- γ mRNA. #, $p < 0.05$ for IL-13 vs IL-13 + IL-12 + IL-18. ND = not done.

expression, they had no effect on IL-13 induction of the SPRR genes. Taken together with the results shown in Fig. 7, these observations suggest that IL-4 induction of IL-12 probably accounts for the failure of IL-4 to strongly induce Ca2T6 gene expression, but is unlikely to account for its failure to strongly induce SPRR gene expression.

Anti-IFN- γ mAb fails to enhance IL-4 induction of the Ca2T6 and SPRR genes

Because IL-4 induces IFN- γ expression through a STAT4-independent mechanism (Fig. 8), it was possible that IL-4-induced IFN- γ , rather than IL-12, is responsible for the failure of IL-4 to strongly induce SPRR gene expression. To evaluate this possibility, mice were inoculated i.t. with IL-4C or IL-13 and injected i.v. with a neutralizing anti-IFN- γ mAb (XMG-6), at a dose that strongly suppresses IFN- γ effects in vivo (42). Anti-IFN- γ mAb enhanced IL-4-induced SPRR1 and SPRR2a gene expression little, if at all, in this experiment and in a second experiment and had no effect on Ca2T6 gene expression (Fig. 9 and data not shown).

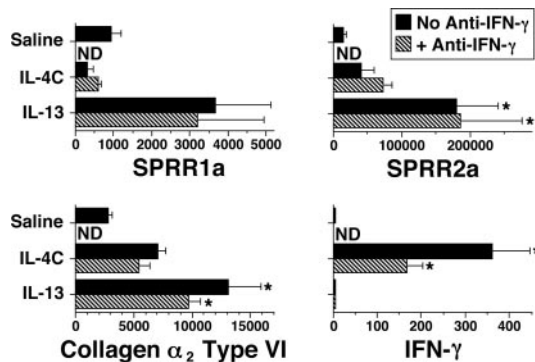


FIGURE 9. IL-4 induction of IL-13-selective genes is not enhanced by anti-IFN- γ mAb. BALB/c mice were inoculated daily i.t. for 4 consecutive days with saline, IL-4C that contained 10 μ g of IL-4, or 1 μ g of IL-13. Mice were also injected i.p. before the first i.t. inoculation with 1 mg of anti-IFN- γ mAb or an isotype-matched control mAb. Mice were sacrificed 1 day after the last i.t. inoculation. Lungs were analyzed for IFN- γ , SPRR1a, SPRR2a, SPRR2b, and Ca2T6 mRNA. SPRR2b levels are not shown because they were too low to be reliably quantitated. ND = not done.

Thus, it appears unlikely that IL-4 induction of IFN- γ is responsible for reduced pulmonary Ca2T6 and SPRR gene expression in mice that have been inoculated i.t. with IL-4.

Discussion

Our studies demonstrate that IL-13 strongly and persistently induces the expression of four genes that are induced poorly and transiently by IL-4. The selective induction of these genes cannot be explained by dose effects: low doses of IL-13 induced expression of these genes considerably more effectively than even high doses of IL-4. Although most of our experiments compared the effects of free IL-13 with the effects of a long-acting form of IL-4 that was prepared by mixing free IL-4 with a neutralizing anti-IL-4 mAb at a 2:1 molar ratio, this difference in IL-4 vs IL-13 preparations did not explain their different effects on gene expression: free IL-4 was no more effective than an equal quantity of complexed IL-4 at inducing gene expression. Our results also indicate that selective induction of the Ca2T6 and SPRR genes by IL-13 is not mediated by a novel IL-13 signaling pathway; both IL-4R α and STAT6 are required for IL-13 induction of these genes. These last results do not conflict with recent evidence for IL-4R α -independent signaling by IL-13, because the putative IL-4R α -independent signaling pathway appears to require the presence of activated CD4⁺ T cells (54–56).

Instead, our results suggest that IL-4 induces both allergy-associated genes, and genes that suppress some elements of allergy. Evidence for this view was provided by three experiments. First, IL-4 initially induced, then suppressed expression of the SPRR2a and SPRR2b genes. Second, pretreatment with IL-4 suppressed the ability of IL-13 to induce the Ca2T6 and SPRR genes. Third, the induction of these genes by IL-4, but not by IL-13, was considerably increased in mice deficient in B cells, T cells, and γ_c , but not in mice deficient only in B cells and T cells. This comparison was made to distinguish the possibility that γ_c is required for IL-4 suppression of the Ca2T6 and SPRR genes because suppression is mediated by B and/or T cells, which are decreased in γ_c -deficient mice, from the possibility that γ_c -related gene suppression is mediated by other effects of γ_c . We favor the possibility that the suppressive effect of IL-4 on Ca2T6 and SPRR gene expression is mediated by non-B, non-T cells that are activated by IL-4 signaling through the type 1 IL-4R (IL-4R α / γ_c). However, we cannot rule

out the possibility that the suppressive effects of IL-4 on gene expression depend, directly or indirectly, on cytokines such as IL-2, IL-7, IL-9, IL-15, or IL-21, which signal through other γ_c -dependent receptors (31, 57–60). For example, IL-4 may activate suppressive effects of NK cells, which require IL-15 for their survival (61).

One non-B, non-T cell type that has already been shown to respond differently to IL-4 vs IL-13 is the DC. Although DCs are reported to express both the type 1 and type 2 IL-4Rs, only IL-4 induces these cells to secrete IL-12 (45). IL-12 is an attractive candidate for suppression of IL-13 induction of the Ca2T6 and SPRR genes because it induces production of IFN- γ and directly, or through IFN- γ , suppresses several Th2 cytokine effects (46, 48). However, only Ca2T6 gene expression was clearly up-regulated in IL-4-inoculated STAT6-deficient vs wild-type mice. Consistent with this, inoculation of wild-type mice i.t. with a mixture of IL-12 and IL-18, which amplifies IL-12 effects (62), stimulated pulmonary production of IFN- γ and suppressed IL-13-induced Ca2T6 gene expression. Furthermore, a potent, neutralizing anti-IFN- γ mAb had little effect on IL-4 induction of Ca2T6 or SPRR gene expression. Thus, it appears likely that effects of IL-4 on non-T, non-B cells other than induction of IL-12 and IFN- γ are responsible for suppression of IL-13-induced SPRR gene expression.

The primary importance of our observations is that they demonstrate that IL-4, in addition to promoting a Th2 response through effects on multiple cell types, limits Th2 responses through effects on non-B, non-T cells that are not restricted to induction of IL-12 or IFN- γ . In contrast, although the proallergic effects of IL-13 are less pervasive than those of IL-4, because IL-13 fails to activate T cells and has limited effects on murine B cells, the proallergic effects of IL-13 are one sided; they are not balanced by suppression of the Th2 response or Th2 cytokine effects. This lack of balance helps to explain why IL-13 appears to be more important than IL-4 as an inducer of the pulmonary inflammation and AHR in mouse models of asthma. The importance of selective induction of the Ca2T6 and SPRR genes by IL-13 as a marker for the different effects of IL-4 and IL-13 should not, however, obscure the possibility that the Ca2T6 and/or SPRR genes may contribute to the IL-13-associated phenotype in murine asthma. In this regard, we have recently observed that allergen inhalation, like IL-13 inhalation, induces SPRR gene expression and that this expression is IL-13 and STAT6 dependent.⁴ Furthermore, SPRR gene expression is primarily restricted in allergen-inoculated mice to pulmonary epithelial cells. Selective effects on this cell type are particularly intriguing because IL-13 induces STAT6-dependent AHR and goblet cell hyperplasia in transgenic mice in which only epithelial cells can activate STAT6 (17). Although the SPRR gene family has previously been associated with epithelial cells, this association has been restricted primarily to squamous epithelial cells (63). The products of these genes contribute to the cellular envelope of squamous epithelial cells, which decreases epithelial permeability and perhaps increases epithelial rigidity (64, 65). In addition, SPRR proteins have been reported to migrate to the nucleus and influence gene expression and cellular differentiation (66). Both of these effects may influence airway responsiveness, and the latter effect has the potential to influence goblet cell differentiation and mucus production. A transgenic approach is currently being taken to directly determine whether increased SPRR

⁴ N. Zimmermann, M. P. Doecker, D. P. Witte, S. M. Pope, P. C. Fulkerson, E. B. Brandt, A. Mishra, N. E. King, N. M. Nikolaidis, M. Wills-Karp, et al. Expression and regulation of small proline-rich protein (SPRR)2 in allergic inflammation. *Submitted for publication*.

gene expression in pulmonary epithelium will promote AHR or goblet cell hyperplasia.

Ca2T6 also has features that make it a reasonable candidate for involvement in the proasthmatic effects of IL-13. These include: 1) evidence that Ca2T6 may promote the migration of inflammatory cells to the lungs by serving as a ligand for adhesion molecules, including Mac-1 (CD11c/CD18) (67); 2) evidence that Ca2T6 ligation of adhesion molecules activates signaling molecules (68); and 3) evidence that Ca2T6 binds oncostatin M, a profibrogenic cytokine (69) that could be involved in the pulmonary fibrosis that develops in IL-13 lung transgenic mice.

However, because reagents required to evaluate SPRR and Ca2T6 protein expression are not available, studies have not yet been performed to determine whether IL-13-induced increases in SPRR and Ca2T6 gene expression result in increased lung expression of the SPRR and Ca2T6 proteins. Such increases in protein levels would be required for increased expression of these genes to have effects on pulmonary structure and function.

In sum, our studies demonstrate that IL-4, in addition to its proallergic effects, has effects that appear to act through multiple mechanisms to limit allergy, and that these latter effects are not shared by IL-13. This novel observation joins with evidence for greater expression of IL-13 than IL-4 in human asthma and mouse models of asthma (35, 70) and for possible signaling by IL-13 through an IL-4R α -independent IL-13R (54–56) to provide an explanation for the greater importance of IL-13 than IL-4 during the effector phase of asthma. In addition, our demonstration of four IL-13-selective genes, including three genes in one family that is expressed by pulmonary epithelial cells in response to allergen immunization, suggests a novel mechanism that may contribute to the allergic phenotype and provide a new potential target for anti-asthma therapy.

Disclosures

The authors have no financial conflict of interest.

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